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## Effect of Heparin on the Decrease in Blood Coagulation Factor Activity Associated with Experimental Endotoxemia

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IN SEPTICEMIA in humans and in experimental animals given endotoxin, a coagulation abnormality has been observed.<sup>1</sup> Gans and Krivit<sup>2</sup> noted a variable decline in fibrinogen and plasminogen levels following administration of *E. coli* endotoxin to dogs. Unlike the Shwartzman reaction in the rabbit, the dog showed no marked deposits of thrombotic material. Despite the absence of histologic evidence of thrombosis, these authors interpreted the decline in fibrinogen concentration as indicative of possible intravascular coagulation. Gans and Krivit<sup>2</sup> and Hardaway *et al.*<sup>3</sup> noted that endotoxin administered to the dog produced a decrease in fibrinogen concentration and platelet count, a prolongation of prothrombin time, and the appearance of a heparin-like substance in the circulating blood.

Recently, West *et al.*<sup>15</sup> analyzed the blood coagulation factor activity in dogs given endotoxin. In all dogs studied a prolongation of the one-stage prothrombin time and/or partial thromboplastin time, a reduction in the activity of factors II, V, VII, VIII, IX, X, XI, and XII, and a slight reduction in fibrinogen concentration was found.

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In 1960, Gans and Krivit<sup>2</sup> pretreated dogs with heparin and administered *E. coli* endotoxin. They noted that heparin pretreatment prevented the decline in fibrinogen levels usually seen in endotoxemia and that the reduction in plasminogen levels seen in heparinized animals was of shorter duration than in nonheparinized animals.

Thomas and Wessler<sup>14</sup> utilizing rabbits, found that heparin in small doses would prevent endotoxin-induced thrombosis in isolated jugular vein segments.

The purpose of experiments here reported is to evaluate the alteration in clotting factor activity associated with endotoxemia in a preheparinized dog.

### Materials and Methods

Thirty-two adult mongrel dogs of both sexes, weighing 10 to 20 Kg., were utilized. The principles of laboratory animal care as established by the National Society for Medical Research were observed. The animals were anesthetized with 30 mg./Kg of pentobarbital sodium intravenously. A polyethylene catheter was placed into the abdominal aorta by way of the right femoral artery and arterial blood pressures were monitored with this catheter using a Sanborn recorder. A second polyethylene catheter was placed in the right ventricle by way of the right femoral vein and was also connected to the recorder. Injections were made by way of a polyethylene catheter in the opposite femoral vein.

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The animals were divided into two groups. Group A consisted of 16 dogs which received *E. coli* endotoxin alone and Group B consisted of 16 dogs which received heparin prior to administration of endotoxin. Endotoxin was prepared from *E. coli*, strain 0111:B<sub>4</sub> according to the method of MacLean and Weil.<sup>10</sup> The dose administered was calculated to be lethal in 60 to 80 per cent of animals. Two dogs, one of group A and one of group B were studied simultaneously. Heparin was administered intravenously as follows: 1) 4 mg./Kg. prior to administration of endotoxin, and 2) 2 mg./Kg. at 2, 4, and 6 hours after administration of the initial dose. Endotoxin was administered to the heparinized animals 15 minutes after the initial dose of heparin.

Blood was collected utilizing a fresh polyethylene catheter for each sample. The catheter was introduced through a cut-down site in the left femoral artery and each was passed further up the artery than the previous catheter to prevent collection of blood from an area of damaged endothelium. Between collections, the artery was clamped above the cutdown site with an atraumatic vascular clamp.<sup>15</sup>

Blood samples for analyses were collected according to the following schedule: 1) a control sample after the dog had been anesthetized and catheters placed but prior to administration of either endotoxin or heparin, 2) from heparinized dogs only, 5 minutes after administration of heparin, and 3) from both heparinized and non-heparinized dogs, 5 minutes after administration of endotoxin and 1, 2, 3, and 4 hours after administration of endotoxin.

The animals were kept in the operating room until the last dose of heparin was administered. The vascular catheters were then removed, incisions closed, and the animals returned to their cages.

All animals which died within the first 48 hours after receiving endotoxin were re-

corded as fatalities. Animals surviving beyond 48 hours were considered survivors. Autopsies were performed on animals which died.

#### Laboratory Examinations

Blood used for one stage prothrombin and partial thromboplastin time was anticoagulated with 3.2% sodium citrate in a ratio of 1 part citrate to 9 parts whole blood. For analytical chemical analyses, platelet counts and hematocrits, blood was collected in a siliconized tube and anticoagulated with EDTA, 0.01 ml. 10% EDTA/1 ml. whole blood. Platelet counts were performed using a phase contrast microscope with appropriate counting chamber.

#### Coagulation Screening Tests

**One-stage prothrombin time.** Reagents and duplicate samples of test plasma and control plasma were brought to 37° C. in a water bath. Then 0.2 ml. of a 1 to 1 mixture of 0.025 M calcium chloride and thromboplastin \* was blown from a pipette into a 10 × 75 mm. plain glass tube containing 0.1 ml. of plasma and a stopwatch was simultaneously started. The tube was tilted in air until a clot formed. The stopwatch was stopped simultaneously with clot formation, and the interval of time required for the clot to form was measured.<sup>2</sup>

**Partial thromboplastin time.** Reagents and duplicate samples of control plasma and test plasma were brought to 37° C. in a water bath. To 0.1 ml. of test and control plasma in separate 10 × 75 mm. test tubes was added 0.1 ml. of a 1 to 1 mixture of a partial thromboplastin \*\* and 2% kaolin. The mixture of plasma, kaolin and partial thromboplastin was incubated for 2 minutes with intermittent, gentle agitation. At

\* For screening tests, "Simplastin" supplied by Warner-Chilcott, Morris Plains, N. J., was used.

\*\* "Platelin" supplied by Warner-Chilcott, Morris Plains, N. J.

TABLE 1. Neutralization of Heparin with Polybrene

Tube No.	1	2	3	4	5	6
Test plasma (heparinized)	0.4 ml.	0.4 ml.	0.4 ml.	0.4 ml.	0.4 ml.	Normal plasma control
Mg. of Polybrene	0	0.01	0.015	0.025	0.035	
P.T. in seconds	28.6	16.3	6.5	7.3	7.4	7.2

the end of this 2 minute incubation, 0.1 ml. of 0.025 M calcium chloride was blown into the mixture and a stop watch started simultaneously. The tube was tilted in air and the interval required for clot formation was timed.<sup>2, 9, 11-13</sup>

**Neutralization of heparin with polybrene.** In order that the one-stage prothrombin and partial thromboplastin times could be performed on heparinized plasma, heparin was neutralized with polybrene.<sup>14</sup> Based on the dose of heparin administered to the experimental animals, the amount present in the plasma was estimated. A series of tubes containing varying amounts of polybrene was set up and aliquots of plasma were mixed with the polybrene. Concentrations of polybrene used extended over a range sufficient to neutralize heparin in the plasma. Table 1 illustrates neutralization of heparin with polybrene in amounts sufficient to neutralize heparin to the extent that the one-stage prothrombin in one of the tubes is within normal limits. Table 1a illustrates neutralization of heparin with polybrene in amounts sufficient to give a normal partial thromboplastin time. It should be noted that slightly more polybrene is required to neutralize heparin sufficiently so that partial thromboplastin time will be within normal limits in one tube.

Fibrinogen was quantitated by precipi-

tation from plasma using sodium sulfite. The precipitate was washed free of other protein and the residual precipitate was quantitated by the biuret technic.

### Results

The one-stage prothrombin time and partial thromboplastin time for both control and heparinized animals are shown in Tables 2 and 3. Fibrinogen concentrations are shown in Table 4 and platelet counts in Table 5. The numbers in the tables are the means of the various measurements before administration of endotoxin and at the various intervals after administration of endotoxin.

One-stage prothrombin and partial thromboplastin times of the nonheparinized animals progressively lengthened over the 4-hr. observation period. In the heparinized group, these examinations remained within normal limits except for the one-stage prothrombin time at 3 hours after administration of endotoxin. At this particular point in time the one-stage prothrombin time was at the upper limits of normal.

Fibrinogen concentration in both groups of animals declined slightly over the 4-hr. observation period. However, relative to the mean of the control sample in each group, the amount by which the fibrinogen concentration declined was the same.

TABLE 1a. Neutralization of Heparin with Polybrene

Tube No.	1	2	3	4	5	6
Test plasma (heparinized)	0.4 ml.	0.4 ml.	0.4 ml.	0.4 ml.	0.4 ml.	Normal plasma control
Mg. of Polybrene	0	0.02	0.025	0.04	0.06	
P.T.T. in seconds	>60	>60	17.3	20.0	34.3	16.5

TABLE 2. Mean Prothrombin Times\*

Nonheparinized Animals						Heparinized Animals					
Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.	Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.
6.7	6.8	7.0	7.8	8.0	8.5	6.7	6.5	7.1	7.1	7.7	7.1

\* In seconds.

\*\* Intervals after endotoxin.

TABLE 3. Partial Thromboplastin Times\*

Nonheparinized Animals						Heparinized Animals					
Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.	Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.
15.6	17.7	18.8	21.6	21.7	24.0	15.1	15.0	15.9	16.6	15.8	14.8

\* In seconds.

\*\* Intervals after endotoxin.

In both groups of animals, there was an appreciable decline in platelet counts 5 minutes after endotoxin, and these gradually returned toward normal levels over the 4-hr. observation period.

#### Discussion

As a measure of coagulation factor activity, one-stage prothrombin and partial thromboplastin times were used in this experiment. These examinations reflect the

activity of all known clotting factors, with the exception of factor XIII.

Results demonstrate that heparinization of a dog prior to administration of endotoxin will reduce the loss of coagulation factor activity. At 3 hours after administration of endotoxin, the one-stage prothrombin time was at the upper limits of variability. At 2 hours after endotoxin, partial thromboplastin time varied very slightly. These changes suggest minimal loss of clot-

TABLE 4. Mean Fibrinogen Concentrations\*

Nonheparinized Animals						Heparinized Animals					
Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.	Con- trol**	5 min.	1 hr.	2 hr.	3 hr.	4 hr.
491	474	398	374	367	357	516	503	517	469	475	437

\* In Mg. %.

\*\* Interval after endotoxin.

TABLE 5. Mean Platelet Counts

Nonheparinized Animals						Heparinized Animals					
Con- trol	5 min.	1 hr.	2 hr.	3 hr.	4 hr.	Con- trol	5 min.	1 hr.	2 hr.	3 hr.	4 hr.
259,620	5,360	104,540	129,410	117,680	127,846	235,120	3,930	78,120	129,720	135,180	139,150

ting factor activity. The nonheparinized animals had a significant prolongation of the clotting times as measured by both these tests at comparable intervals indicating a much greater loss of coagulation factor activity.

Although previous authors have stated that heparin will prevent the decline in fibrinogen levels following endotoxin administration,<sup>4</sup> a significant difference between fibrinogen levels of heparinized and nonheparinized animals could not be established in this experiment.

Heparin at this dose level did not prevent reduction in the number of circulating platelets. Previous experiments in this laboratory when massive doses of heparin were given, showed a similar inability of heparin to prevent thrombocytopenia.

Morphologic studies revealed similar changes in heparinized and nonheparinized animals. Both groups had moderate to severe vascular congestion of the small bowel, liver, and lungs with less severe congestion of other organs.

An additional significant result of this experiment was that heparinization did not alter mortality. Although defects in blood coagulation were largely prevented by heparinization, a larger percentage of heparinized animals died within the first 48 hours after administration of endotoxin than did nonheparinized animals. These results were of interest because previous work of other investigators had shown that heparin would reduce mortality from 100% in control animals to 48% in preheparinized animals.<sup>6</sup> Still other authors demonstrated that heparin would prevent mortality with LD 50 doses of endotoxin, but that this agent would not prevent mortality when the amount of endotoxin administered was increased to LD 100.<sup>7</sup>

The mechanism by which heparin prevented loss of clotting factor activity is not defined specifically by this experiment. However, two major theories are offered.

First, heparin by its anticoagulant action, may prevent utilization of clotting factors in intravascular fibrin formation and second, heparin has been shown to be an inhibitor of proteolytic enzyme activity which may well explain the results observed here.<sup>8</sup>

The anticoagulant action of heparin is dependent upon its capacity to inhibit plasma thromboplastin formation and to prevent the conversion of fibrinogen to fibrin. Since in this experiment, the partial thromboplastin times of heparinized animals remain within normal limits, it would appear that coagulation factors which contribute to plasma thromboplastin formation were not utilized. From this evidence, one can infer that these animals did not generate plasma thromboplastin *in vivo*.

With the exception of calcium all known clotting factors are proteins. Excess activity of proteolytic enzymes, such as fibrinolysin or trypsin, could inactivate one or more of clotting factors. From other experiments in this laboratory, an increase in proteolytic activity in plasma of animals subjected to endotoxemia has been demonstrated and associated with a loss of clotting factor activity. However, the specific enzyme or enzymes responsible for this proteolysis have not been identified. Since heparin may inhibit proteolytic enzymes including trypsin, it might protect against the loss of clotting factor activity through this action.

### Summary

1. Preheparinized and nonheparinized mongrel dogs were given endotoxin, and clotting factor activity was studied using the one-stage prothrombin time and partial thromboplastin time.

2. Loss of clotting factor activity in heparinized animals was largely prevented, whereas control animals lost significant amounts of clotting factor activity.

3. Preheparinization did not affect mortality.

4. The possible mechanisms by which heparin may prevent loss of clotting factor activity are discussed.

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